ISSN 1682-296X (Print)
ISSN 1682-2978 (Online)

Bio Technology

ANSInet
Asian Network for Scientific Information
308 Lasani Town, Sargodha Road, Faisalabad - Pakistan

Biotechnology 10 (5): 408-414, 2011
ISSN 1682-296X / DOI: 10.3923/biotech.2011.408.414

In vitro ACE-inhibitory and Antioxidant Activities of the Casein Hydrolysates Subjected to Plastein Reaction with Addition of Three Extrinsic Amino Acids

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Abstract: Plastein reaction was used in the present work to modify casein hydrolysates in the presence of extrinsic amino acids to reveal its impacts on two bioactivities. Casein was hydrolyzed by alcalase to a degree of hydrolysis of 12.4%. The prepared hydrolysates exhibited *in vitro* inhibition on Angiotensin-I-converting Enzyme (ACE) and scavenging activity on DPPH radical. The prepared hydrolysates were then modified by alcalase-catalyzed plastein reaction in the presence of one of three extrinsic amino acids (leucine, valine or phenylalanine). At fixed 35% (w/w) substrate concentration and 6 h reaction time, other conditions were optimized as 0.6 mol/mol amino acid addition, 3 kU g^{-1} peptides alcalase addition and 30°C reaction temperature. The modified hydrolysates exhibited better ACE inhibition for its IC_{50} value decreased from 42.2 to 21.0-25.1 mg mL^{-1}. The scavenging activity on DPPH (hydroxyl) radical or reducing power of the modified hydrolysates was also better than that of original casein hydrolysates. The results show that alcalase-catalyzed hydrolysis of casein coupled with plastein reaction is capable of preparing casein hydrolysates with better ACE inhibition and antioxidant activity.

Key words: Casein hydrolysates, plastein reaction, amino acids, ACE-inhibitory activity, antioxidant activity

INTRODUCTION

Hypertension and cardiovascular disease were serious threats to human health (Lopez-Fandino *et al*., 2006). Angiotensin-I-converting Enzyme (ACE) is physiologically important in the regulation of blood pressure. Inhibition of ACE can result in a lowing of blood pressure (Koike *et al*., 1980). Synthetic drugs for treatment hypertension include captopril and lisinopril but exhibit some side effects such as drowsiness, headache, excessively low blood pressure (Fitzgerald *et al*., 2004). Therefore, natural and safe ACE inhibitors have attracted attention of both food and medical scientists. Food protein hydrolysates have ACE-inhibitory activity (Li *et al*., 2004; Miguel *et al*., 2007; Pihlanto *et al*., 2008) and milk proteins are considered to be particularly good sources of ACE-inhibitory peptides (Lopez-Fandino *et al*., 2006). ACE-inhibitory peptides encrypted in intact milk proteins can be liberated by enzymatic hydrolysis or fermentation. When milk protein preparations were hydrolyzed by five proteases, thermolysin was a very good enzyme to release ACE-inhibitory peptides (Otte *et al*., 2007). Two ACE-inhibitory peptides IPP and VPP, with IC_{50} values of 5 and 9 μmol L^{-1}, respectively, were separated from fermented milk products or milk protein hydrolysates (Fitzgerald *et al*., 2004; Nakamura *et al*., 1995). Optimized hydrolysis conditions were used to improve ACE-inhibitory activity of protein hydrolysates (Guo *et al*., 2009).

There are strong evidences that reactive oxygen species and free radicals play an important role in many degenerative diseases such as cancer, atherosclerosis and diabetes (Beckman and Ames, 1998). Formation of free radicals, such as superoxide anion radical and hydroxyl radical, is an unavoidable consequence of respiration in aerobic organisms. These radicals are very unstable and reactive, leading to cell or tissue injury. The body has its own defense system against free radicals but might not be enough to completely prevent the oxidative damage. Food supplements containing antioxidants may help the body to reduce oxidative damage. Several studies reported the free radical-scavenging activity of proteins or peptides from various food sources, such as milk (Pihlanto, 2006), egg (Davalos *et al*., 2004), soybean (Pyo and Lee, 2007; Hang and Zhao, 2011), potato (Wang and Xiong, 2005) and rice (Sirirat and Jelena, 2010).

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Plastein reaction, as a reversal of enzymatic proteolysis was applied to prepare a higher molecular protein-like substance. There were three different mechanisms in early thoughts, which were supposed as transpeptidation (Combes and Lozano, 1992), physical forces (Andrews and Alichanidis, 1990) and condensation (Yamashita *et al*., 1976a). Recent work also suggested that several pathways might play a role in the plastein reaction simultaneously (Stevenson *et al*., 1999). Plastein reaction can improve biological value and functional properties of food proteins, mask the undesired flavor of protein hydrolysates and especially provide an approach to form new protein sources. Soybean proteins (Ashley *et al*., 1983; Yamashita *et al*., 1976b), milk proteins (Cheeseman, 1981) and fish proteins (Kolakowski *et al*., 1997; Raghunath and McCurdy, 1991) had been used as plastein reaction substrates.

It is not known yet that whether plastein reaction has influence on the two activities of protein hydrolysates treated. The purpose of this work was to study the impacts of alcalase-catalyzed plastein reaction on the two activities of casein hydrolysates in the presence of three extrinsic amino acids.

MATERIALS AND METHODS

Materials: Casein, N-(3-[2-furyl]acryloyl)-L-phenylalanylglycylglycine (FAPGG) and rabbit lung acetone powder were purchased from Sigma-Aldrich Co.(St. Louis, US). Alcalase with an activity of 100 kU g^{-1} was purchased from Novozyme China (Tianjing, China). Other reagents used were of analytic grades. Highly purified water prepared with Milli-Q PLUS (Millipore Corporation, New York, NY., US) was used for the preparation of all buffers and solutions.

Preparation of casein hydrolysates: Casein solution (20 g, on dry basis) was dissolved in 200 mL water and adjusted to pH 8.5 by adding few drops of 2 mol L^{-1} NaOH. Alcalase solution (1 kU mL^{-1} water) was prepared immediately prior to use. After withdrawal of a 15 mL sample (zero-time sample), the hydrolysis process was started by the addition of 1 mL of Alcalase solution to the remaining casein solution (giving approximately 1 kU g^{-1} proteins) with continuous stirring and carried out at 55°C. Some hydrolyzed samples of 15 mL were withdrawn after 1, 2, 3, 4, 5, 6 and 7 h of hydrolysis, respectively. All samples were heated at 100°C for 15 min to inactivate the enzyme. After cooling to room temperature, all samples were centrifuged at 11000×g for 20 min and the supernatant (casein hydrolysates) collected was evaluated for its degree of hydrolysis, ACE-inhibitory and antioxidant activity. The hydrolysates exhibited higher ACE-inhibitory and antioxidant activity was thus prepared as the substrate of plastein reaction.

Table 1: Independent variables and their ranges selected in the response surface methodology

	Independent variables		
Levels	Amino acid addition (X_1, mol mol^{-1})	E/S ratio (X_2, kU g^{-1} peptides)	Reaction temperature (X_3, °C)
-1.618	0.05	1.6	13
-1	0.20	3.0	20
0	0.40	5.0	30
+1	0.60	7.0	40
+1.618	0.75	8.4	47

Optimization of plastein reaction of casein hydrolysates: Plastein reaction conditions for casein hydrolysates in the presence of extrinsic amino acid were studied by employing response surface methodology with a central composite design. In the design, amino acid addition (mol mol^{-1} free amino groups of casein hydrolysates) (X_1), alcalase addition (E/S ratio, kU g^{-1} peptides) (X_2) and reaction temperature (°C) (X_3) were chosen as independent variables (Table 1). Casein hydrolysates concentration and reaction time were fixed at 35% (w/w) and 6 h, respectively. The decreased amount of free amino groups in the reaction mixture was taken as the dependent variable (viz. response) (Y). The response was calculated by subtracting the content of free amino groups of the modified hydrolysates after reaction from that of the substrates before reaction. The second-order polynomial coefficients were calculated by using the Design Expert software Version 7.0 (Stat-Ease Inc., Minneapolis, MN, USA). Second-degree polynomials as below were used to predict the response:

$$Y = \beta_0 + \Sigma_{i=1}^{3}\beta_i x_i + \Sigma_{i=1}^{3}\beta_{ii} x_i^2 + \Sigma_{i=1}^{2}\Sigma_{j=i+1}^{3}\beta_{ij} x_i x_j$$

where, Y was the dependent variable, β_0, β_i, β_{ii} and β_{ij} were coefficients estimated by the model and x_i, x_j were levels of the independent variables.

The polynomial equation was expressed as three-dimensional surface plots to visualize the relationship between the response and the levels of each variable and to deduce the optimal conditions.

Assaying of the degree of hydrolysis: An OPA (o-pthaldialdehyde) method (Spellman *et al*., 2003) was used to measure the free amino groups or to calculate the degree of hydrolysis (DH) of casein hydrolysates. Nitrogen content of casein or the hydrolysates was determined by the Kjeldahl method. A factor of 6.38 was used to calculate protein or peptide content. The DH of the hydrolysates was calculated by the method given by Adler-Nissen (1979).

Determination of ACE-inhibitory activity: The ACE-inhibition assay was performed as that described by Murray *et al.* (2004), with FAPGG as substrate and extract of rabbit lung acetone powder as the ACE source.

The concentration of the hydrolysates needed to inhibit the ACE by 50% (IC_{50}) was determined by assaying variously diluted hydrolysates and plotting the ACE inhibition percentage as a function of peptide concentration (Shalaby *et al.*, 2006).

Assaying scavenging activity on DPPH radical: The scavenging activity of the analysis sample on DPPH radical was measured with the method of Nsimba *et al.* (2008) in the spectrophotometer and ethanol was used as control.

Measurement of reducing power: The reducing power of the analysis sample (5 mg peptides mL^{-1}) was measured at 700 nm as the method of Yildirim *et al.* (2001) in the spectrophotometer. Higher absorbance of the assaying mixture indicated higher reducing power of sample solution.

Assaying scavenging activity on hydroxyl radical: The assay was carried out by using the method described by Li *et al.* (2008).

Statistical analysis: All data were expressed as Mean±SD from at least three independent trials. Differences between the mean values of multiple groups were analyzed by one-way analysis of variance (ANOVA) and Duncan's multiple comparison test with SPSS 13.0 for Windows (SPSS Inc., Chicago, IL, US). The level of confidence required for significance was set at $p<0.05$. Microsoft Excel version 2003 software (Microsoft Corporation, Redmond, WA, US) and SPSS version 13.0 software (SPSS Inc., Chicago, IL, US) were used to report the data.

RESULTS AND DISCUSSION

Preparation of casein hydrolysates: Alcalase was employed in the present work to prepare casein hydrolysates and carry out plastein reaction. ACE-inhibitory activity or scavenging activity on DPPH radical of the hydrolysates prepared over 7 h hydrolysis periods were measured and shown in Table 2. As hydrolysis reaction was progressed during first 5 h, the DH of the hydrolysates increased from 0 to 12.4%. At the same time, the inhibitory (or scavenging) activity of the hydrolysates at 50 μg mL^{-1} (or 1 mg mL^{-1}) increased from 5.4 (or 21.4) to 50.5 (or 40.8) %. Thereafter, the DH or activity of the hydrolysates showed a little increase but the scavenging activity showed an increase trend. When hydrolysis time was 6 h, both the inhibitory activity (51.2%) and scavenging activity (41.8%) of the hydrolysates were the highest ($p<0.05$). Based on these facts, casein hydrolysates of DH 12.4% were prepared as substrate of plastein reaction. Analysis showed that the IC_{50} value for the hydrolysates to inhibit ACE was 42.2 μg mL^{-1}.

Table 2: Hydrolysis of casein by alcalase and the degree of hydrolysis (DH), ACE-inhibitory activity or scavenging activity on DPPH radical of the prepared hydrolysates[1]

Hydrolysis time (h)	DH (%)	ACE-inhibitory activity [2] (%)	Scavenging activity on DPPH radical [2] (%)
0	0	5.4±1.1a	21.4±0.6a
1	7.9±0.6a	33.6±1.1b	37.0±0.7b
2	9.8±0.5b	36.5±1.0c	39.3±0.6c
3	10.9±0.5c	45.4±2.8d	39.5±0.6c
4	11.6±0.4cd	48.7±2.0d	40.7±0.5d
5	12.4±0.5d	50.5±1.6d	40.8±0.4d
6	12.4±0.4d	51.2±2.3d	41.8±0.3e
7	12.5±0.4d	50.7±1.8d	42.0±0.5e

[1]Different letters after the values in same column indicate that one-way ANOVA of the means is different significantly ($p<0.05$). [2]The final peptide content used in ACE-inhibitory activity or scavenging activity on DPPH radical was 50 μg mL^{-1} or 1 mg mL^{-1}, respectively

Enzymatic hydrolysis can release bioactive peptides from milk protein precursors and alcalase are often used for this purpose. It seems that hydrophobic amino acids at each of the three C-terminal positions of the peptides are favorable to their ACE inhibition (Li *et al.*, 2004; Lopez-Fandino *et al.*, 2006). Casein is rich in proline, tryptophane, tyrosine and phenylalanine, which give casein hydrolysates opportunity to have these amino acids at C-terminal position. Otte *et al.* (2007) treated sodium caseinate with thermolysin, the obtained hydrolysates had IC_{50} value of 95 μg mL^{-1}. Miguel *et al.* (2009) hydrolyzed bovine casein with pepsin, the prepared hydrolysates had IC_{50} value of 52.8 μg mL^{-1}. Mao *et al.* (2007) also hydrolyzed yak casein with various proteases including alcalase and the scavenging activity of the prepared hydrolysates on DPPH radical were less than 80% at 2.5 mg mL^{-1}. The hydrolysates we prepared exhibited similar ACE inhibition to the hydrolysates of Miguel *et al.* (2007) but superior inhibition to the hydrolysates of Otte *et al.* (2007). The hydrolysates also had a comparable scavenging activity on DPPH radical to the hydrolysates prepared by Mao *et al.* (2007).

Optimal conditions of plastein reaction for casein hydrolysates: When leucine was added to the casein hydrolysates and the independent variables shown in Table 1 were studied with response surface methodology, a quadratic model with statistical significance was obtained as follow.

$$Y= -76.76638+355.20764X_1+20.11282X_2+1.86278X_3 -28.86118X_1X_2+1.79988X_1X_3-252.71503X_1^2- 1.03322X_2^2-0.052625X_3^2$$

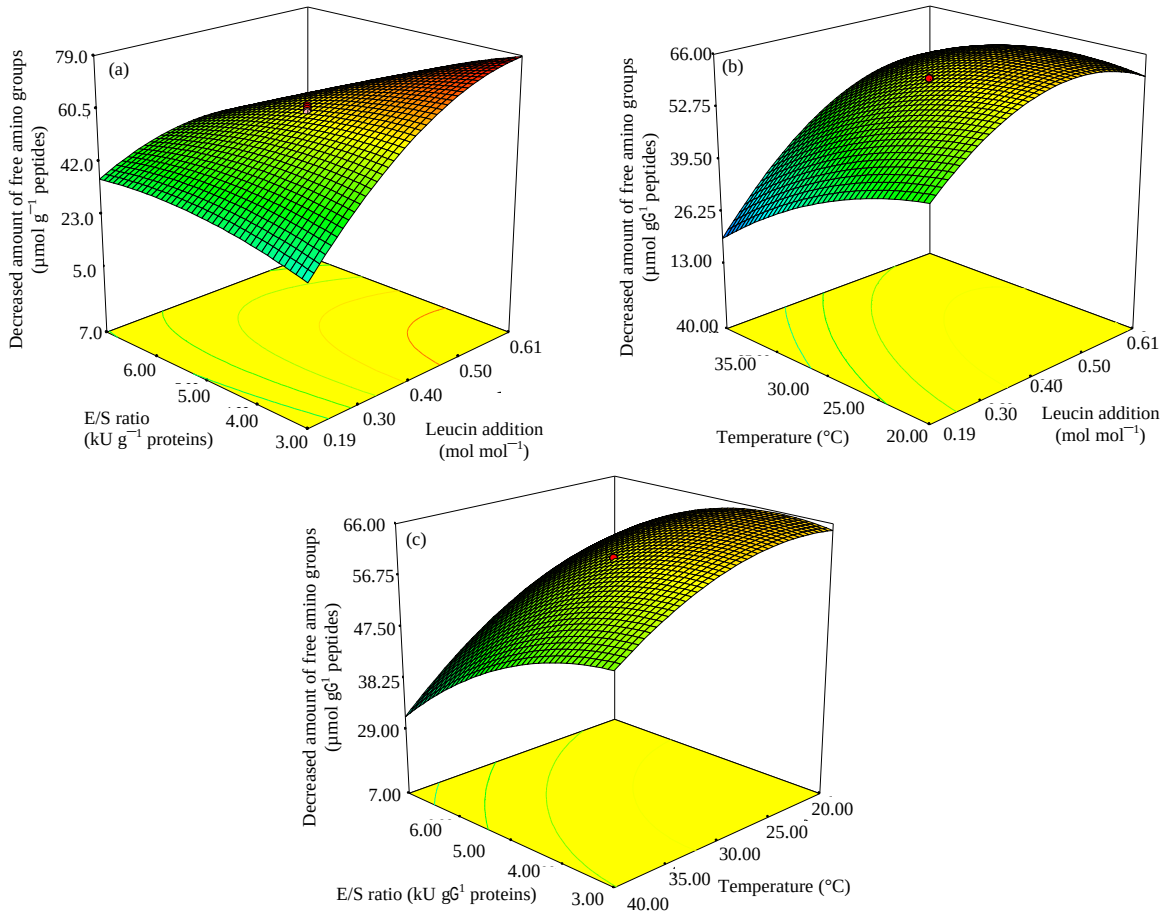

Fig. 1 (a-c): Response surface graphs for the decreased amount of free amino groups in the plastein reaction mixture in the presence of leucine as a function of: (a) E/S ratio and addition level of leucine (reaction temperature at the central of its level); (b) addition level of leucine and reaction temperature (E/S ratio at the central of its level); (c) reaction temperature and E/S ratio (addition level of leucine at the central of its level)

To assess the effects of independent variables on the decreased amount of free amino groups of the modified hydrolysates, three response surface plots were generated (Fig. 1). From Fig. 1a, it can be seen that the decreased amount of free amino groups of the modified hydrolysates was increased as the increase of both leucine addition and alcalase addition. Figure 1b indicates that the decreased amount of free amino groups of the modified hydrolysates was increased with the increase of leucine addition but decreased as the increase of reaction temperature. In Fig. 1c, it is shown that the decreased amount of free amino groups of the modified hydrolysates was increased with the increase of alcalase addition and decrease reaction temperature. Three reaction conditions studied all showed influences on the decreased amount of free amino groups of the modified hydrolysates. A higher alcalase addition, a higher leucine addition but a lower reaction temperature might be favorable to the plastein reaction of casein hydrolysates. X_1, X_2 and X_3 were thus selected at +1, +1 and -1 level. Plastein reaction conditions were leucine addition of 0.6 (mol mol^{-1}), E/S ratio of 3 kU g^{-1} peptides and reaction temperature of 20°C, respectively, with the fixed substrate concentration of 35% (w/w) and reaction time of 6 h.

The practical study also showed that if valine or phenylalanine was added to the casein hydrolysates, the optimized plastein reaction conditions were consistent with the optimized conditions mentioned above (detailed data not shown here).

Plastein reaction usually occurs at substrate concentration 30-50% by weight (Lozano and Combes, 1991; Pallavicini *et al*., 1980). The fixed substrate

Table 3: Effects of plastein reaction on *in vitro* ACE-inhibitory and antioxidant activity of the Modified Casein Hydrolysates (MCH)[1]

Sample	Amino acid added[3]	IC_{50}[4] (μg mL^{-1})	SA on DPPH radical[4] (%)	Reducing power	SA on OH radical[4] (%)
CH[2]	-	42.2±0.4e	41.8±0.6a	0.406±0.004a	16.9±2.3a
MCH 1	-	32.2±0.7d	50.1±1.5b	0.456±0.005b	50.4±0.9c
MCH 2	Leu	25.1±0.7c	49.8±0.9b	0.455±0.003b	51.8±1.7c
MCH 3	Val	21.0±0.2a	49.6±0.4b	0.452±0.002b	45.3±0.8b
MCH 4	Phe	23.3±0.5b	50.8±1.6b	0.456±0.005b	47.6±0.9b

[1]The peptide concentration used for the evaluation of scavenging activity on DPPH or OH radical was 1 or 5 mg mL^{-1} while that used for the evaluation of reducing power was 5 mg mL^{-1}. Different letters after the values in same column indicate that one-way ANOVA of the means is different significantly ($p<0.05$). [2]CH, casein hydrolysates. [3]Leu, leucine; Val, valine; Phe, phenylalanine. [4]IC_{50}, peptide concentration needed to inhibit ACE activity by 50%; SA: Scavenging activity

concentration or selected higher leucine addition in the present study are consistent with this conclusion. Plastein reaction is an exothermic reaction and a low reaction temperature is beneficial to peptide synthesis (Fujimaki *et al*., 1971). The selected lower reaction temperature in the present study also shared consistence to this finding. Besides these conditions mentioned, a higher enzyme addition should be selected as plastein reaction is an enzyme-catalyzed reaction.

***In vitro* ACE-inhibitory and antioxidant activities of the modified casein hydrolysates:** When leucine or valine or phenylalanine was added to casein hydrolysates, three modified hydrolysates (MCH 2-4) were prepared with the selected conditions. A control modified hydrolysates (MCH 1) was also prepared but without amino acid addition. The MCH 1-4 and the original casein hydrolysates were evaluated for their ACE-inhibitory and antioxidant activity listed in Table 3.

Compared to original casein hydrolysates, MCH 1-4 exhibited much better ACE-inhibitory or antioxidant activity ($p<0.05$), indicating that plastein reaction is an effective approach to enhance the two activities of casein hydrolysates. MCH 2-4 also exhibited higher ACE-inhibitory activity (or lower IC_{50}) (esp. valine was added) than MCH 1 ($p<0.05$) (IC_{50} 21.0-25.1 vs. 32.2). The result states that extrinsic amino acid added in plastein reaction can improve further the ACE-inhibitory activity of casein hydrolysates. MCH 1-4 had same scavenging activity on DPPH radical (ranging from 49.6 to 50.8%, $p>0.05$) and same reducing power (ranging from 0.452 to 0.456, $p>0.05$). On the other hand, MCH 1-2 showed some higher scavenging activity on hydroxyl radical than MCH 3-4 (50.4-51.8% vs. 45.3-47.6%, $p<0.05$). Totally, three added extrinsic amino acids exhibite no significant impact on the antioxidant activities of the modified hydrolysates ($p>0.05$), except for the scavenging activity on hydroxyl radical. Based on these results, plastein reaction in the presence of extrinsic amino acid might be an applicable ways to give casein hydrolysates a higher ACE-inhibitory and antioxidant activity.

The casein hydrolysates prepared by Miguel *et al*. (2009), Otte *et al*. (2007) and Contreras *et al*. (2009) had ACE inhibition with IC_{50} values of 52.8, 95 and 22.19-60.85 μg mL^{-1}, respectively. Compared to these hydrolysates prepared, the prepared MCH 2-4 exhibited a stronger inhibition on ACE. Suetsuna *et al*. (2000) separated a peptide from casein hydrolysates, which gave an IC_{50} 98 μmol L^{-1} (equal to 2.45 mg mL^{-1}) on DPPH radical. The hydrolysates from fish muscles by pepsin, pancreatin or thermolysin had scavenging activity on DPPH radical with an IC_{50} from 4.88 to 9.91 mg mL^{-1} (Nakajima *et al*., 2009). The prepared MCH 2-4 showed scavenging activity on DPPH radical about 50% at 1 mg peptides mL^{-1}. This result means that the prepared MCH 2-4 had a higher scavenging activity than the hydrolysates in these two works.

CONCLUSION

The present study shows that the optimized alcalase-catalyzed plastein reaction can enhance the ACE-inhibitory or antioxidant activity of casein hydrolysates prepared by alcalase. Addition of one of three extrinsic amino acids (leucine, valine or phenylalanine) in reaction system resulted in the modified hydrolysates much higher ACE-inhibitory activity. The scavenging activity on DPPH (or hydroxyl) radical and the reducing power of the modified hydrolysates were not impacted or enhanced by the added amino acids. Alcalase-catalyzed hydrolysis of casein coupled with plastein reaction can prepare casein hydrolysates with much better ACE-inhibitory and antioxidant activity.

ACKNOWLEDGMENTS

This study was funded by the National Natural Science Foundation of China (No. 30972132) and the Innovative Research Team of Higher Education of Heilongjiang Province (No. 2010td11). The authors also thank anonymous reviewers and the editors for their constructive and valuable works to our study.

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